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Title:	Diagnostic Testing for COVID-19 Bridging Study for CDC EUA assays vs CDC Multiplex N1 FAM, N2 SUN, RNAse P ATTO 647 Assays
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Los Alamos National Laboratory
Biological Agent Testing Laboratory

Diagnostic Testing for COVID-19
Bridging Study for
CDC EUA assays vs
CDC Multiplex N1 FAM, N2 SUN, RNAse P ATTO 647 Assays

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Executive Summary

This report describes testing performed by LANL's Biological Agent Testing Laboratory (BATL) to validate modifications to the CDC EUA 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel (EUA-CDC-nCoV-IFU). BATL intends to implement the modification to increase thoughput for daily testing. BATL validated the original component, CDC designed primers and probes purchased from IDT (Cat # 10006770) and the new component, CDC assays designed with new Reporter dyes allowing the assays to be multiplexed, IDT (Cat # 10006830, 10006831, 10006823, 10006833, 10006834, 10007050, 10006836, 10006837, 10007062)

1.0 Introduction and Purpose

LANL's Biological Agent testing Lab (BATL) performs testing for SARS-CoV-2 for LANL employees, and provides surge capacity testing for the State of New Mexico. To increase the testing capabilities of BATL, we conducted a bridging study to validate proposed changes to the CDC-EUA and establish equivalency between the original component for assay probes and primers to be used in a singleplex format and the same primers and probes designed with different reporter dyes to allow a multiplex assay format. The 2019-nCoV real-time RT-PCR diagnostic panel consists of singleplex assays, wherein one individual PCR signature is evaluated in one reaction well on a 96-well plate. BATL needed to increase PCR efficiency and cost by moving to a multiplex assay. In the multiplexed assay, the three individual PCR signatures of the 2019-nCoV can be evaluated in one individual reaction well. This report summarizes the methods BATL used to validate the higher throughput alternatives and the results demonstrating equivalency.

2.0 Evaluation Process

2.1 Range Finding

BATL selected a known positive clinical sample collected and provided by the New Mexico Department of Health Scientific Laboratory Division (NM DoH SLD) as a sample spike, that fell within a Ct Range of 20-25. A set of known negative samples were pooled together to provide the sample matrix. The detection range of the original CDC Emergency Use Authorized (EUA) assay was determined using a dilution series produced by spiking the positive sample into the pooled negative sample matrix. Serial dilution ranges were prepared depending on the initial Ct, using the pooled negative clinical samples as a diluent. A dilution series of five concentrations were run in triplicate beginning with an approximate expected Ct of 30.

For example:
If Initial Ct = 20.
1:10 _expected Ct 23.3

1:100_ expected Ct 26.6
1:1000_ expected Ct 30
1:10000_ expected Ct 33.3
1:100000_ expected Ct 36.6
1:1000000_ expected Ct 40
1:10000000_ expected Ct 43.3

Once the range was determined using the original extraction method and assays, we then performed the Limit of detection (LOD) study described next, to determine equivalency between the extraction kits.

2.2 Limit of Detection (LOD)

The purpose of this experiment was to identify the lowest concentration for which a set of triplicate reactions generate positive results, using a dilution series of the virus in clinical matrix. This would be the target dilution for LOD. The dilution window was further tested with the new assays. Three concentrations consisting of the target dilution identified during the range finding as well as one 3-fold dilution above and below the identified target dilution, were tested. Each dilution was extracted 4 times using the QIAamp extraction kit, and each extract was run 5 times for a total of 20 replicates of each concentration. Multiplex assays were evaluated using the Thermofisher TaqPath™ 1-Step RT-qPCR Master Mix, CG. The RNAse P singleplex reaction was tested in 10 replicates at each of the three concentrations providing 30 replicates to compare to the multiplexed RNAse P assay. The two assays were considered to have equivalent performance if the resultant Limit of Detection (LoD) for the multiplex assay was the same as the LoD for the unmodified authorized test (i.e., ≤3xLOD)

The following two conditions were tested.

1. Qiagen kit with original CDC assay
2. Qiagen kit with new multiplex CDC assay

2.3 Equipment and Supplies

2.3.1 Instrumentation and equipment

- Rainin Pipettes
    - Rainin pipettes were used to mix and transfer reagents and samples. Calibrations are performed annually (or as needed) by Rainin,

- Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument with SDS version 1.4 software
    - This instrument was used to perform PCR testing on clinical specimens. An Operational and Performance Qualification was performed by a qualified ThermoFisher/ABI technician. Testing performance was evaluated with controls included in every test plate run on the instruments during Assay verification and actual testing.

2.3.2 Reagents and Materials

2.3.2.1 RNA Extraction

For each of the kits listed below, CDC has confirmed that the external lysis buffer is effective for inactivation of SARS-CoV-2. BATL has been using one of these options for viral RNA extraction.

- QIAmp DSP Viral RNA Mini Kit: 50 extractions (61904)
- QIAamp Viral RNA Mini Kit: 250 extractions (52906)

2.3.2.2 Primers and probes

CDC has determined certain commercially available kits of primers and probes may be used with the procedures in CDC-006-00019, Revision: 04, effective 12/01/2020. These kits are listed at https://www.cdc.gov/coronavirus/2019-nCoV/lab/index.html. BATL used the following approved probes and primers;

Manufacturer	**Product Name**	**Catalog No.**	**Acceptable Lots**
Integrated DNA Technologies (IDT) www.idtdna.com (800) 328-2661	2019-nCoV Kit, 500 rxn	10006606	0000500383 0000504847 0000505594 0000505969 0000507295 0000507509 0000508150 0000508785 0000509402 0000510344 0000510908 0000511393 0000512209 0000512692 0000513132

Integrated DNA Technologies (IDT) www.idtdna.com (800) 328-2661	2019-nCoV Kit, 1000 rxn	10006770	0000515825

BATL tested the equivalency of the following assay in a multiplex format;

Manufacturer	**Product Name**	**Catalog No.**
Integrated DNA Technologies (IDT) www.idtdna.com (800) 328-2661	nCOV_N1 Forward Primer	10006830
Integrated DNA Technologies (IDT) www.idtdna.com (800) 328-2661	nCOV_N1 Reverse Primer	10006831
Integrated DNA Technologies (IDT) www.idtdna.com (800) 328-2661	nCOV_N1 Probe (FAM)	10006823
Integrated DNA Technologies (IDT) www.idtdna.com (800) 328-2661	nCOV_N2 Forward Primer	10006833
Integrated DNA Technologies (IDT) www.idtdna.com (800) 328-2661	nCOV_N2 Reverse Primer	10006834
Integrated DNA Technologies (IDT) www.idtdna.com (800) 328-2661	nCOV_N2 Probe (SUN)	10007050
Integrated DNA Technologies (IDT) www.idtdna.com (800) 328-2661	Rnase P Forward Primer	10006836
Integrated DNA Technologies (IDT) www.idtdna.com (800) 328-2661	Rnase P Reverse Primer	10006837
Integrated DNA Technologies (IDT) www.idtdna.com (800) 328-2661	Rnase P Probe (ATTO 647)	10007062

2.3.2.3 Positive control

In the context of limited quantities of testing supplies during this public health crisis, based on the best available evidence and in consultation with outside experts, the US

Food & Drug Administration (FDA) has determined that certain materials may be used as positive controls:

Control	Manufacturer	Product Name	Catalog Number
N1/N2 Positive Control	Integrated DNA Technologies (IDT), 800-382-2661	2019-nCoV_N_Positive Control	10006625
HS_RPP30 Positive Control	Integrated DNA Technologies (IDT), 800-382-2661	HS_RPP30_Positive Control	10006626

2.3.2.3 RT-PCR Enzyme Mastermix

The following options are available. BATL used the Promega GoTaq Probe 1-step RT-qPCR System

Reagent	Quantity	Catalog No.
Quantabio qScript XLT One-Step RT-qPCR ToughMix	100 x 20 µL rxns (1 x 1 mL)	95132-100
	2000 x 20 µL rxns (1 x 20 mL)	95132-02K
	500 x 20 µL rxns (5 x 1 mL)	95132-500
Quantabio UltraPlex 1-Step ToughMix (4X)	100 x 20 µL rxns (500 µL)	95166-100
	500 x 20 µL rxns (5 x 500 µL)	95166-500
	1000 x 20 µL rxns (1 x 5 mL)	95166-01K
Promega GoTaq® Probe 1- Step RT-qPCR System	200 x 20 µL rxns (2 mL)	A6120
	1250 x 20 µL rxns 12.5 mL	A6121
Thermofisher TaqPath™ 1-Step RT-qPCR Master Mix, CG	1000 reactions	A15299
	2000 reactions	A15300

2.4 Data Analysis

2.4.1 Reagent acceptance procedures, criteria and controls

The reagent acceptance procedures, criteria and controls for the original system have been provided by the CDC. They are documented in Standard Analytical Method, SAM 927, “Quality Control of CDC Reagents.” A SAM for multiplex reagent acceptance was developed and approved by the project manager prior to the start of testing.

2.4.2 Test plate acceptance procedures, criteria and controls

A negative template control (NTC) and the appropriate positive control (nCoV_PC, or RPP30_PC) were included in each amplification and detection run.

2.4.3 Range finding Data

Table 1 shows Ct values obtained for the dilution series prepared using a single positive sample that had a SLD reported Ct value of 24. Each dilution was extracted with the QIAamp Viral RNA mini kit and run using the CDC-EUA assays. The target dilution to use for the equivalency study was identified to be 1:10,000 based on the qualitative detection criteria provided in the Instructions for Use for the CDC-EUA assay.

Sample	N1 Avg	N1 Std	N1 Detects		N2Avg	N2 Std	N2 Detects		RP Avg	RP Std	RP Detects
SLD sample (ct24) 1:100	29.51	0.10	3/3		31.08	0.18	3/3		28.07	0.10	3/3
SLD sample (ct24) 1:1,000	32.94	0.51	3/3		34.02	0.37	3/3		28.30	0.08	3/3
SLD sample (ct24) 1:10,000	35.73	1.43	2/3		37.70	0.39	2/3		28.15	0.02	3/3
SLD sample (ct24) 1:100,000	N/A	N/A	1/3		N/A	N/A	1/3		28.15	0.06	3/3
SLD sample (ct24) 1:1,000,000	N/A	N/A	1/3		N/A	N/A	0/3		28.01	0.13	3/3

Table 1 – Range finding to determine the target dilution to use for equivalency testing

2.4.4 Limit of Detection (LOD)

Table 2 shows the comparison of the performance of the multiplex assay format of the CDC assay with the original authorized component used in the CDC-EUA assay. Three concentrations consisting of the target dilution identified during the range finding, (1:10,000 of spiked sample with an initial Ct of 24), and one 3-fold dilution above and below the identified target dilution, were tested. The data shows that the LOD between the approved CDC assays and the IDT Multiplexed CDC assays are equivalent with the multiplexed assays having a slightly lower Ct value than the CDC approved assays. Qualitatively the results are equivalent with the multiplex format showing a higher detection number from 20 replicates.

Singleplex CDC assays										
Sample	**Avg Ct N1**	**Stdev Ct N1**	**N1 Detects**		**Avg Ct N2**	**Stdev Ct N2**	**N2 Detects**	**Avg Ct Rp**	**Stdev Ct N2**	**N2 Detects**
SLD sample (ct24) 1:3,000	34.41	0.80	20/20		35.37	0.81	20/20	25.76715	0.16907188	10/10
SLD sample (ct24) 1:10,000	36.02	0.91	18/20		37.10	1.09	20/20	27.62415	0.13495679	10/10
SLD sample (ct24) 1:30,000	37.50	0.93	9/20		38.23	0.93	9/20	26.59323	1.16972022	10/10

Multiplex CDC assay										
Sample	**Avg Ct N1**	**Stdev Ct N1**	**N1 Detects**		**Avg Ct N2**	**Stdev Ct N2**	**N2 Detects**	**Avg Ct RP**	**Stdev Ct RP**	**RP Detects**
SLD sample (ct24) 1:3,000	31.90	1.27	20/20		33.37	0.73	20/20	25.48	0.56	20/20
SLD sample (ct24) 1:10,000	33.52	1.29	20/20		35.20	1.10	20/20	26.25	1.84	20/20
SLD sample (ct24) 1:30,000	35.09	1.53	15/20		37.37	0.90	14/20	26.30	1.02	20/20

Table 2 – Comparison of CDC EUA singleplex format of assay to multiplex format

3.0 References

1. **CDC. Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens from Persons for Coronavirus Disease 2019 (COVID-19).** https://www.cdc.gov/coronavirus/2019-nCoV/lab/guidelines-clinical-specimens.html.
2. **IATA. Shipping Regulations for UN 3373 Biological Substances**, Category B. https://www.iata.org/contentassets/b08040a138dc4442a4f066e6fb99fe2a/dgr-61-en-pi650.pdf.
3. **CDC. Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19).** https://www.cdc.gov/coronavirus/2019-nCoV/lab/lab-biosafety-guidelines.html
4. **CDC. CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel**, CDC-006-00019, Revision: 03, effective 3/30/2020. https://www.fda.gov/media/134922/download
5. **CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel Acceptable Alternative Primer and Probe Sets**. https://www.cdc.gov/coronavirus/2019-ncov/downloads/List-of-Acceptable-Commercial-Primers-Probes.pdf.

6. **FDA. FAQs on Diagnostic Testing for SARS-CoV-2**. https://www.fda.gov/medical-devices/emergency-situations-medical-devices/faqs-diagnostic-testing-sars-cov-2#whatif
7. **IDT. 2019 Novel Coronavirus (COVID-19) Virus Detection Assay Kit Reagents**. https://www.idtdna.com/pages/landing/coronavirus-research-reagents#references.
8. **RNAdvance Viral XP instructions for Use**. https://www.beckman.com/search#q=c59543&t=coveo-tab-techdocs
9. **QIAGEN. QIAamp Viral RNA Mini Handbook.** https://www.qiagen.com/us/resources/resourcedetail?id=c80685c0-4103-49ea-aa72-8989420e3018&lang=en
10. **Thermo Fisher. Applied Biosystems. Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument Instructions for Use**. https://assets.thermofisher.com/TFS-Assets/LSG/manuals/4406991.PDF,